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Original Research Paper

Association of volatile terpenoids and their biosynthetic genes in high temperature stress tolerance in tomato (*Solanum lycopersicum* L.)

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ABSTRACT

One of the major limiting factors for the production of tomato is the high temperature stress. Plants are capable of sensing the stress early and produce the terpenoid compounds which may contribute for tolerance or act as a signaling compound to trigger the expression of many tolerant genes. Therefore, the present study was conducted to understand the variability in the production of volatile terpenoid compounds in tolerant (IIHR-2841) and susceptible (IIHR-2914) genotypes and the related gene expression under high temperature stress conditions. Genotypes IIHR-2841 and IIHR-2914 were exposed to high temperature (40±2°C) at early flowering stage using polytunnel. Volatile compounds were extracted and identified using SPME-GC-MS. Higher expression of the terpenoid synthase genes and increased release of terpenoids were observed in the tolerant genotype under stress. Expression of β-Caryophyllene synthase (TPS12) and β-Phyllandrene synthase (TPS20) showed a remarkable 2-fold increase at 9th day of temperature stress in tolerant genotype IIHR-2841 whereas; IIHR-2914 did not show upregulation.

Keywords: Abiotic stress, GC-MS, gene expression, high temperature, terpenoids, tomato

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second-most important vegetable in the world after potato. The production is affected by many biotic and abiotic stresses. Ideal temperature for tomato cultivation is between 25°C and 30°C during day and 20°C during night (Camejo et al., 2005). Maintenance of productivity during temperature stress depends upon the tolerance ability of the plant. Various plant responses through metabolites contribute to the tolerance mechanism (Arbona et al., 2013). Terpenoids are one of such groups which confer tolerance against high temperature in plants. Under natural conditions, tomato emitted constitutive mono- and sesquiterpene volatiles, which are highly enhanced by both abiotic and biotic stress conditions (Jansen et al., 2009). In leaf, terpenoid emissions are accompanied by two genes β-phellandrene synthase gene (TPS20) and (E)-β-caryophyllene/α-humulene synthase gene (TPS12) (Falara et al., 2011). Plants volatile isoprenoids act as stress-protective agents by their antioxidative capacities (Vickers et al., 2009) which are transformed by membrane fluidity (Behnke et al., 2007). Emission of isoprenoids increases with increasing temperature (Valolahti et al., 2015) due to the immediate effects of temperature on the activity of rate limiting enzymes, terpene synthases (Li & Sharkey, 2013). In plant cells, all terpenoids are synthesized by terpene synthases (TPSs), which are localized in the cytoplasm and chloroplast to produce sesquiterpenes and monoterpenes respectively (Wang et al., 2018). However, there are no reports on variation in emission of volatiles compounds in contrasting genotypes for thermotolerance in tomato. The aim of the present study is to examine the variations in terpenoids emission and the gene expression of β-phellandrene and (E)-β-caryophyllene synthases in the tolerant and susceptible genotypes of tomato under high temperature stress conditions.

MATERIALS AND METHODS

The present study was carried out at the Division of Basic Sciences, ICAR-Indian Institute of Horticultural Research (ICAR-IIHR), Bengaluru during 2016-17 and 2017-2018. The experimental site is located at 13°582 N latitude, 78° E longitude and 890 m above

mean sea level. The genotypes were selected using temperature induction response (TIR) technique for high temperature tolerance as Biradar et al. (2019). Tomato seeds of IIHR-2841 (high temperature tolerant) and IIHR-2914 (high temperature susceptible) was sown in pro-trays and seedlings were transplanted in the field after 35 days of sowing. The experiment was laid out in a completely randomized block design with five replications. At the early flowering stage (30 days after transplanting), the temperature stress (40±2°C) was imposed using polytunnel (L 25 ft x H 6 ft x W 10 ft) and the other set was maintained outside the polytunnel as control. Leaf samples were collected from both control and temperature stress treatments at second, fifth and ninth day after imposition of temperature stress, early morning around 8:00 AM wrapped in aluminum foil dipped immediately in liquid nitrogen and brought to the lab for further processing.

Extraction of leaf volatiles

Adsorption of volatiles was done in the field without removing the leaves by using solid phase micro extraction (SPME). Twigs of leaves were inserted into the polypropylene vacuum desiccators with silicone rubber caps and SPME fiber was inserted into the desiccators to adsorb the head-space volatiles for 2 hours. Later fiber was removed and injected into a GC-MS for separation and identification of compounds. GC-MS analysis was performed on Varian-3800 gas chromatograph coupled with Varian 4000 GC-MS-MS ion trap mass selective detector. The compounds were identified by comparing the retention index and compared the spectra using two spectral libraries available as Wiley and NIST-2007.

RNA isolation, quantification, and cDNA synthesis

Total RNA was isolated from leaves and apical buds using the trizol method (Chomczynski & Mackey, 1995). RNA quantification was done by measured using Nanodrop spectrophotometer (NanoDrop-1000 spectrophotometer). RNA integrity was examined by gel electrophoresis on 1.2% agarose gel. cDNA synthesis was done by using Himedia cDNA synthesis kit, the reverse transcription reaction product can be directly used in PCR applications.

Gene expression analysis by QPCR

Primers were designed using primer quest tool from integrated DNA technologies (IDT) software (http://eu.idtdna.com/Primerquest/Home/Index) with default parameters (Table 1). To compare between temperature treated and control expression levels, the function $\Delta\Delta^{CT}$ was done using the equation $\Delta\Delta^{CT} = \Delta^{CT}$ (treatment) - Δ^{CT} (control). Relative expression values were calculated as $2^{-\Delta CT}$ normalizing against the reference gene (Livak & Schmittgen, 2001). Gene specific primers were used for expression studies using ABI7500 PCR system from Applied Biosystems (AB).

RESULTS AND DISCUSSION

Terpenoids profiling by GC-MS

Based upon the morphological and physiological data, two contrasting genotypes were selected for volatile profiling (Lokesha et al., 2019). More than 45 major volatile compounds were identified in both genotypes under control as well as high temperature stress conditions. High temperature increased the quantity of volatile compounds in both the genotypes whereas most of the volatiles were higher in tolerant compared to susceptible genotypes. The major groups of volatile compounds found in tomato genotypes were monoterpenes, sesquiterpenes, hydrocarbons, alcohols, aldehydes, ketones and a few others (Table 2 & Fig. 1).

In present study, monoterpenes (70-85%) were more prominent than sesquiterpene (1-15%). Among the monoterpenes, β-phellandrene was dominant, followed by α-terpinene, α-phellandrene, 2-carene, 1,3,8-p-menthatriene, Δ-3-carene, α-pinene, 2-β-pinene. Under high temperature, the emission of β-phellandrene

Table 1 : Details of primers used for qRT-PCR

Target gene	Primer sequence	
	Forward (5^{l}-3^{l})	**Reverse (5^{l}-3^{l})**
TPS-20	GAATTATCCGATGCTCGTCTC	GCATAGTCATCCCACCTTTC
TPS-12	GCATAGTCATCCCACCTTTC	CGACAGACTCAACCAGTAAC
Actin	ATAACCGCATCAGGTCTCCA	CCGAAGTTACGGATCCATTT

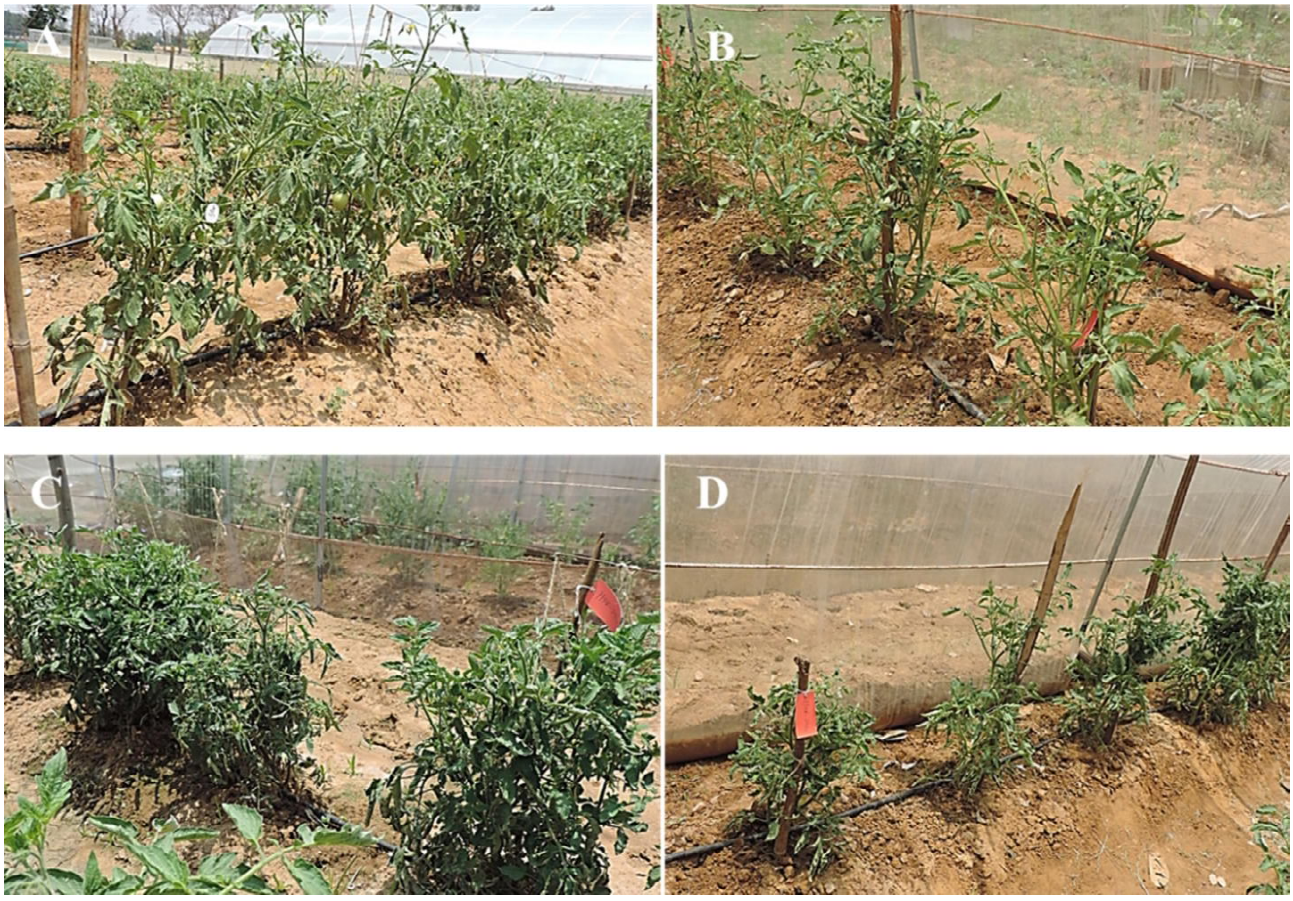

Fig. 1 : Tomato plants of IIHR-2841, control (A), stress (B) (high temperature tolerant) and IIHR-2914 control (C), stress (D) (high temperature susceptible)

increased in both the genotypes compared to control. Under stress β-phellandrene emission in IIHR-2841 was 5.98 ng/100 g as compared to 5.05 ng/100 g in IIHR-2914. α-Phellandrene emission in IIHR-2841 0.31 ng/100 g as compared to 0.24 ng/100g in IIHR-2914. Under high temperature conditions, increased emissions of terpenoids were reported due to the enhanced activities of terpene biosynthesis enzymes (Loreto et al., 2006; Loreto & Schnitzler, 2010; Duan et al., 2019). The terpenoids emission is controlled mainly by temperature and light (Dindorf et al., 2006; Loreto et al., 2007) and these emissions increase exponentially with temperature (Tarvainen et al., 2005; Helmig et al., 2007).

With regard to sesquiterpenoids, *trans*-caryophyllene was dominated in both the genotypes followed by δ-elemene, ε-muurolene, bicyclogermacrene, β-guaiene, patchoulane. IIHR-2841 recorded *trans*-caryophyllene emission of 0.96 ng/100 g as compared to IIHR-2914 with an emission of 0.51 ng/100 g under high temperature stress. Among the hydrocarbons, p-cymene and E, E-2, 6 - Dimethyl - 1, 3, 5, 7-octatetraene increased under stress conditions in both the genotypes. Volatile production is considered to be proportional to temperature, the higher the temperature, the greater the production of volatiles (Fallik et al., 1997). Fatty acids serve as the precursor for alcohols which could be generated through lipoxygenase pathway of unsaturated linoleic and linolenic acids (Perez et al., 1999). The results revealed that alcohol group, the compounds like viridiflorol, carveol and khusilol were also increased under stress in both the genotypes. Copolovici et al. (2012) demonstrated that temperature stress results in a major enhancement of terpenoid emissions. However, while constitutive and heat enhanced monoterpene emissions were dominated by β-phellandrene in both the studies, sesquiterpene emissions were dominated by α-humulene (Copolovici et al., 2012) and by trans-caryophyllene (Pazouki et al., 2016). Such differences may be due to different cultivars used in these studies.

Table 2 : Content of volatile compounds from two contrasting tomato genotypes leaves subjected to high temperature stress (9th day) obtained by SPME/GC-MS analysis

RT	Volatile Compounds	KI value	IIHR-2914 Control ng/100g	IIHR-2914 Stress ng/100g	IIHR-2841 Control ng/100g	IIHR-2841 Stress ng/100g
	Hydrocarbons					
1.786	3-methyl-Pentane	570	0.4276	0.0318	0.0040	0.0000
3.281	Toluene	762	0.0072	0.0041	0.0131	0.0086
9.462	Benzene	975	0.0360	0.0804	0.1288	0.1288
12.312	p-Cymene	1025	0.0307	0.2277	0.1268	0.2756
5.936	o-Cymene	1028	0.0055	0.0019	0.0072	0.0040
15.022	E, E-2,6-Dimethyl-1,3,5,7-octatetraene	1134	0.0006	0.0039	0.0024	0.0055
51.296	Cyperene	1332	0.0016	0.0000	0.0000	0.0000
		Total	**0.5091**	**0.3498**	**0.2822**	**0.4224**
	Monoterpenes					
7.649	α-Pinene	932	0.0448	0.0201	0.0550	0.0438
10.409	2- β-Pinene	978	0.0009	0.0771	0.0088	0.0393
25.946	2-Carene	1001	0.0136	0.0967	0.0689	0.1137
11.198	α- Phellandrene	1005	0.0486	0.2428	0.2130	0.3082
13.586	δ-3-Carene	1009	0.0000	0.0848	0.0000	0.0447
10.773	α- Terpinene	1019	0.3355	0.3068	0.9760	0.6255
12.539	β- Phellandrene	1034	1.3519	5.0502	3.9789	5.9849
14.187	γ- Terpinene	1062	0.0035	0.0389	0.0127	0.0362
15.786	α-Terpinolene	1085	0.0009	0.0199	0.0064	0.0293
13.251	1,3,8-p-menthatriene	1111	0.0129	0.0618	0.0341	0.0902
		Total	**1.8126**	**5.9991**	**5.3538**	**7.3157**
	Sesquiterpenes					
31.46	δ-Elemene	1347	0.0000	0.0525	0.0027	0.0921
32.759	Patchoulane	1393	0.0000	0.0094	0.0032	0.0263
38.395	Junipene	1413	0.0003	0.0082	0.0004	0.0055
41.202	Aromadendrene	1423	0.0010	0.0262	0.0044	0.0115
33.872	β-Guaiene	1423	0.0035	0.0326	0.0343	0.0273
37.968	ε-Muurolene	1430	0.0000	0.0183	0.0021	0.0438
36.51	Trans-Caryophyllene	1440	0.0030	0.5071	0.0418	0.9550
37.517	α-Bergamotene	1456	0.0000	0.0095	0.0000	0.0050
38.7	α-Humulene	1459	0.0004	0.0282	0.0055	0.0026
47.831	Thujopsene	1469	0.0004	0.1207	0.0073	0.1925
35.639	γ- Himachalene	1478	0.0000	0.0324	0.0025	0.0163
34.906	β-Selinene	1483	0.0000	0.0170	0.0030	0.0184
31.214	bicyclogermacrene	1492	0.0044	0.0167	0.0019	0.0307
37.968	α-Muurolene	1494	0.0000	0.0117	0.0006	0.0184
45.82	Eremophilene	1530	0.0000	0.0084	0.0000	0.0194
		Total	**0.0139**	**0.9404**	**0.1158**	**1.5548**

	Alcohol					
1.56	2-methyl-2-Propanol	526	0.0224	0.0000	0.0000	0.0000
16.069	α- isopropylanisole	1117	0.0344	0.0172	0.0113	0.0393
16.624	Carveol	1220	0.0042	0.0252	0.0090	0.0398
33.205	Viridiflorol	1594	0.0010	0.0240	0.0105	0.0548
51.571	Khusilol	1633	0.0000	0.0301	0.0054	0.0297
		Total	**0.0619**	**0.0965**	**0.0362**	**0.1636**
	Aldehyde and Ketone					
31.717	Trans-bicyclo [4.4.0] decan-1-ol-3-one	1423	0.0000	0.0432	0.0198	0.1100
32.375	(2E)-2-Methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butenal	1586	0.0003	0.0058	0.0030	0.0000
		Total	**0.0003**	**0.0490**	**0.0228**	**0.1100**
	Other compounds					
5.067	Chlorobenzene	837	0.0000	0.0068	0.0251	0.0162
22.77	Cantharidine	944	0.0016	0.0203	0.0075	0.0212
26.553	(2E,3Z)-2-Ethylidene-6-methyl-3, 5-heptadienal	1182	0.0048	0.0130	0.0144	0.0273
30.253	Ascaridole	1238	0.0000	0.0301	0.0111	0.0665
46.229	Caryophyllae oxide	1537	0.0020	0.0867	0.0105	0.0809
		Total	**0.0084**	**0.1568**	**0.0686**	**0.2121**

High temperature increases isoprenoids emissions (Valolahti et al., 2015), activity of rate controlling enzymes like terpene synthases (Li & Sharkey, 2013) and biosynthesis and emission of biogenic volatile organic compounds (BVOCs) (Loreto & Schnitzler, 2010). Mild temperature stress resulted in declining constitutive monoterpene emission, whereas both long-term temperature stresses increased the emission of green leaf volatiles and glucosinolate volatiles (Kask et al., 2016). In both abiotic and biotic stress conditions the composition of emitted terpenoids were modified leading to a high proportion of induced mono- and sesquiterpenes in the total emissions (Penuelas & Staudt, 2010). The genotypes producing higher quantum of volatiles (IIHR-2841) can effectively resist temperature stress by reducing the oxidative stress effects. Monoterpenoids were found to be the major group of terpenoids responsible for the high temperature tolerance in tomato when compared to other groups of volatiles.

Terpene synthase genes (TPS 12 and TPS20)

In plants, terpenoids group mainly includes monoterpenes and sesquiterpenes. Terpene synthases (TPSs) are the key enzymes in the terpene biosynthesis pathway. Tolerant genotype IIHR-2841 did not show any significant difference in the TPS12 gene expression at the early stages of stress (Fig. 2), whereas, at 9th day stress showed a remarkable increase (3.85 folds). But, the susceptible genotype IIHR-2914 showed down regulation of the TPS12 gene during early stages of stress, but slowly it increased 5th day onwards and showed meager 1.51 folds increased expression at 9th day of stress. Similarly, in IIHR-2841, TPS20 expression increased by 1.6 folds at early stages of stress (2 days) but increased to about 4.14 folds at 9th day of temperature stress (Fig. 3). The genotype IIHR-2914 did not show any up regulation during early stages of stress but slowly the expression was increased as stress increased and reached to 1.50 folds at 9th day of stress.

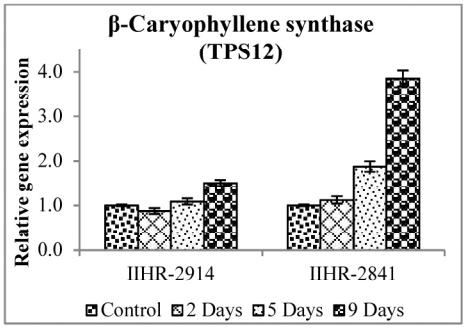

Fig. 2: β-Caryophyllene synthase (TPS12) gene expression pattern under different days of temperature stress in two contrasting tomato genotypes

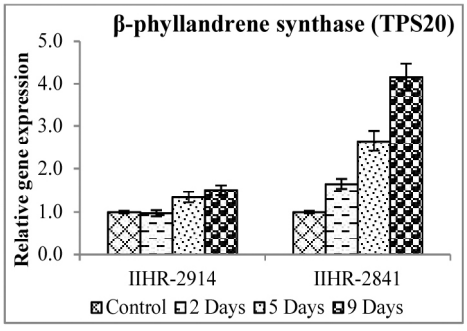

Fig. 3: β-Phyllandrene synthase (TPS20) gene expression pattern under different days of temperature stress in two contrasting tomato genotypes

The emission of some terpene volatiles is highly dependent on temperature, light and oxidative stresses (Holopainen & Gershenzon, 2010). Synthesis of monoterpenes was positively enhanced with temperature by enzymatic activity of monoterpene synthases (Niinemets, 2010). Under natural conditions the terpenoids were continuously released at slow rate (Jansen et al., 2009, Maes & Debergh, 2003), whereas these emissions are highly enhanced under both biotic and abiotic stresses (Copolovici et al., 2012, Maes & Debergh, 2003, Jansen et al., 2009). In the case of leaf terpenoid emissions, two genes are likely of special significance, β-phellandrene synthase gene (TPS20) and (*E*)-β-caryophyllene/α-humulene synthase gene (TPS12) (Falara et al., 2011)). As the plastidial monoterpene synthesis typically relies on substrates provided by photosynthetic metabolism (Rosenkranz & Schnitzler, 2013) both are often correlated (Niinemets et al., 2002). Pazouki et al. (2016) reported that the terpenoid synthesis is highly sensitive to heat stress and mainly controlled at the gene level while severe stress leads to non-recoverable declines in foliage physiological functions and gene expression levels.

CONCLUSION

In the current investigation, more than 45 major volatile compounds were identified in both genotypes under control and high temperature stress. Under high temperature stress conditions, the emission of β-phellandrene increased substantially in both the genotypes compared to control. In particular, volatile isoprenoids act as stress-protective agents through their antioxidative effects and by modifying membrane fluidity of plant cells upon stress. In plants, terpenoids groups mainly include monoterpenes and sesquiterpenes. Terpene synthases (TPSs) are the key enzymes in the terpene biosynthesis pathway. Tomato is a constitutive mono- and sesquiterpene emitter under natural conditions and these emissions are highly enhanced by both biotic and abiotic stresses. These stress-induced volatiles are typically playing important role as lipid-soluble antioxidants in plant stress resistance. Higher expression levels of both the TPS genes were observed in the tolerant genotype indicating the differential control of expression between the tolerant and the susceptible genotypes.

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